

## ***Microneura* is a junior synonym of *Protoneura* (Zygoptera, Coenagrionidae)**

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*Microneura caligata* (Hagen in Selys, 1886) is an endangered damselfly presently known from five localities in the central mountains of Cuba. The precise systematic position of this species within the former Neotropical Protoneuridae has been the subject of debate, with previous results from a phylogenetic analysis based on morphology suggesting that the genus *Microneura* should be placed within the genus *Protoneura*. Here, we used mitochondrial and nuclear DNA sequencing to disentangle the taxonomic status of this species. Our results show that *Microneura* belongs to the *Protoneura* clade, thus making *Microneura* a junior synonym of *Protoneura*. Finally, we provide notes on some observations of emergence and ovipositing behaviour of this species.

**Keywords:** Odonata; dragonfly; Protoneuridae; Neotropics; endemism; molecular taxonomy

### **Introduction**

The high number of endemic species found in the West Indies makes this a priority region for the conservation of odonates in the Neotropical region (Paulson, 2004). Cuba, the largest of the islands within the West Indies, harbours six endemic odonate species out of the 84 species recorded for this island (Torres-Cambas, Lorenzo-Carballa, Ferreira, & Cordero-Rivera, 2015; Torres-Cambas, Trapero-Quintana, et al., 2015).

One of the most emblematic Cuban endemics is *Microneura caligata* (Hagen in Selys, 1886), which has been recorded in only five localities at the Guamuhaya Mountains, in the central region of the island (Paulson, 2004; Ramos & Rodríguez, 2012; Torres-Cambas, Trapero-Quintana, et al., 2015; Trapero Quintana & Torres Cambas, 2008; Trapero-Quintana & Naranjo López, 2003; Westfall & May, 2006). Its restricted distribution and the threats to its habitat have supported its classification as Endangered by the IUCN (Paulson & von Ellenrieder, 2006).

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*Microneura* is currently considered a monotypic genus, although its validity as a genus different from *Protoneura* is still uncertain. The species was named by Hagen (1867), and later described by the same author in Selys (1886) from an adult male. Hagen distinguished this genus from *Protoneura* based on a number of characteristics which included a more robust thorax, a shorter abdomen, larger legs and more hairs on the legs. Gundlach (1888) mentioned the species as *Protoneura (Microneura) caligata* and gave his own description of the species, but he did not justify this genus assignment. Calvert (1919) compared Gundlach's with Selys' description and concluded that the two coincided, and thus he referred to the species as *Microneura caligata* in his publication. Westfall (1964) also adopted the name *Microneura caligata* and he also made the main contribution to the knowledge on the biology of this species until now: he described the larva and teneral male, illustrated the male cerci and female ovipositor, and described its habitat and oviposition behaviour. In the most comprehensive work on Cuban odonates, Alayo (1968) included a key to the Protoneurinae from the island and distinguished the genus *Microneura* from *Protoneura* by having a more oblique nodus, long legs and compressed femurs. Westfall and May (2006) reproduced Westfall's (1964) description of the species, illustrated the female mesostigmal plates, and distinguished between the larvae of *Microneura* and *Protoneura*, with the former having a different coloration pattern, relatively shorter and wider prementum, longer antennae and more prominent eyes. Garrison, von Ellenrieder, and Louton (2010, p. 367) included as unique characters for *M. caligata* "the male femora flattened and expanded at distal end, with black apical half covered with dense white pruinosity". Finally, the most complete phylogenetic study of the Neotropical Protoneurinae up to date, based on morphological characters (Pessacq, 2008) showed that *Microneura caligata* forms a monophyletic clade with the other four *Protoneura* species included in that study, and thus it would be possible to consider it a species of *Protoneura* rather than a distinct genus. In the same paper, *Protoneura* was recovered as polyphyletic, without any unique characters or synapomorphies found for the genus.

Here, we use nuclear and mitochondrial DNA sequencing to disentangle the taxonomic status of *M. caligata*, and we provide also some notes on emergence and ovipositing behaviour of this emblematic species.

## Material and methods

### Sample collection

Samples of *Microneura caligata* ( $n = 4$ ) were collected at the stream Vegas Grandes in Topes de Collantes, Cuba (21°54'51"N, 80°1'20"W), during the course of fieldwork to sample odonates in this area of the island on 16 and 17 June 2012.

Samples of the *Protoneura* type species *P. capillaris* Rambur, 1842 ( $n = 4$ ) of *P. sanguinipes* Westfall, 1987 ( $n = 4$ ), *P. viridis* Westfall (1964) ( $n = 4$ ), *Neoneura maria* Scudder, 1866 ( $n = 4$ ) and *N. amelia* Calvert 1903 ( $n = 4$ ) were collected during different field trips to Cuba, Dominican Republic, Mexico and Jamaica (Table 1). Adult individuals were captured using a hand net and preserved in 90–100% ethanol at 4°C prior to DNA extraction. For *M. caligata* we used a non-invasive sampling technique, in which we took a leg from each captured specimen (Thompson, Hassall, Lowe, & Watts, 2011).

### DNA extraction and sequencing

Total genomic DNA was extracted from legs of collected specimens using the QiagenDNeasy® Blood and Tissue kit (Qiagen, Venlo, the Netherlands), following the supplementary protocol

Table 1. Specimens included in this study. For each individual are listed the species name, individual ID, sex, collection dates and localities, and the GenBank accession numbers for the COII and PRMT loci.

Species	Individual ID	Sex	Collection date	Locality	Country	GenBank accession numbers		References
						COII	PRMT	
<i>Protoneura caligata</i>	MiCal1	F	17/06/2012	Topes de Collantes	Cuba	KT956144	KT956162	This study
	MiCal2	M	16/06/2012	Topes de Collantes	Cuba	KT956145	KT956164	This study
	MiCal3a	F	17/06/2012	Topes de Collantes	Cuba	KT956146	KM276633	This study / Ferreira, Lorenzo-Carballea, et al. (2014)
	MiCal3b	M	17/06/2012	Topes de Collantes	Cuba	KT956147	KT956163	This study
<i>P. capillaris</i>	Pcal01	M	23/08/2012	Dos Bocas	Cuba	KT956141	n.a.	This study
	Pcal02	M	23/08/2012	Dos Bocas	Cuba	KT956142	n.a.	This study
	Pcal03	M	24/08/2012	Dos Bocas	Cuba	n.a.	KM276631	This study / Ferreira, Lorenzo-Carballea, et al. (2014)
	Pcal04	M	23/08/2012	Dos Bocas	Cuba	KT956143	KT956168	This study
	Pcal05	F	23/08/2012	Dos Bocas	Cuba	n.a.	KT956167	This study
<i>P. sanguinipes</i>	Psan01	M	09/05/2012	La Isabela	Dominican Republic	KT956148	KM276634	This study / Ferreira, Lorenzo-Carballea, et al. (2014)
	Psan02	M	09/05/2012	La Isabela	Dominican Republic	KT956149	n.a.	This study
	Psan03	M	09/05/2012	La Isabela	Dominican Republic	KT956150	KT956165	This study
	Psan04	M	09/05/2012	La Isabela	Dominican Republic	KT956151	n.a.	This study
<i>P. viridis</i>	Pvir01	M	15/05/2012	Reach Falls	Jamaica	KT956137	KT956166	This study
	Pvir03	M	15/05/2012	Reach Falls	Jamaica	KT956138	KM276632	This study / Ferreira, Lorenzo-Carballea, et al. (2014)
	Pvir04	M	15/05/2012	Reach Falls	Jamaica	KT956139	n.a.	This study
	Pvir05	M	15/05/2012	Reach Falls	Jamaica	KT956140	n.a.	This study
<i>N. amelia</i>	Nam01	M	14/06/2009	Veracruz	Mexico	KT956154	n.a.	This study
	Nam02	M	14/06/2009	Veracruz	Mexico	KT956155	n.a.	This study
	Nam03	M	14/06/2009	Veracruz	Mexico	KT956156	KM276629	This study / Ferreira, Lorenzo-Carballea, et al. (2014)
	Nam04	M	14/06/2009	Veracruz	Mexico	KT956157	KM276630	This study / Ferreira, Lorenzo-Carballea, et al. (2014)
<i>N. maria</i>	Nmar01	M	17/06/2012	Topes de Collantes	Cuba	KT956158	n.a.	This study
	Nmar02	M	23/08/2012	Dos Bocas	Cuba	KT956160	n.a.	This study
	Nmar03	M	23/08/2012	Dos Bocas	Cuba	KT956161	KM276628	This study / Ferreira, Lorenzo-Carballea, et al. (2014)
	Nmar04	M	23/08/2012	Dos Bocas	Cuba	KT956159	n.a.	This study
<i>Ischnura hastata</i>	IhX35	M	16/05/2012	Dunedin, St Elizabeth	Jamaica	KT956153	KM276612	This study / Ferreira, Lorenzo-Carballea, et al. (2014)
	IhD32	M	10/05/2012	La Vega	Dominican Republic	KT956152	KM276611	This study / Ferreira, Lorenzo-Carballea, et al. (2014)

of the manufacturer for purification of DNA from insects. The mitochondrial COII gene was amplified using the primers COII-F-SF (5'-TAA AGT TTT AAT GTG GCA GA-3') and COII-R-SF (5'-AGT ATT TCG TTA YTT TAC TAT-3') (Ferreira, Velo-Antón, et al., 2014). Additionally, we sequence a fragment of the arginine methyltransferase (PRMT) nuclear gene, with the primers ARG\_F2 (5'-TGC CAC CTT CCT AAT AGA GCT C-3') and ARG\_R3 (5'-TGC CGC CAA GGC TGG AGC ATC-3') (Ferreira, Lorenzo-Carballa, et al., 2014).

All PCR amplifications were carried out in an Applied Biosystems 2720 Thermal cycler (Applied Biosystems, Foster City, CA, USA) in 10 µl reaction volumes containing 1 × DreamTaq® Green PCR Master Mix (Thermo Scientific, Massachusetts, USA), 1 × BSA (10 mg ml<sup>-1</sup>), 1.5 mM MgCl<sub>2</sub>, 0.2 pmol of each primer and ~10–50 ng of DNA. PCR amplification profiles were: 9 min at 95°C, 35 cycles of [95°C for 30 s; 54°C for 30 s; 72°C for 45 s], and 5 min at 72°C. PCR products were purified with the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany), and sequenced bidirectionally using BigDye v.3.1 chemistry (Applied Biosystems) and capillary electrophoresis on an ABI3730XL sequencer (Applied Biosystems) at Macrogen Laboratories (Seoul, Korea).

### Phylogenetic analyses

Forward and reverse sequences for each individual were assembled and manually edited in Geneious version 7.1.7 created by Biomatters (available from <http://www.geneious.com/>). Consensus sequences were aligned with MUSCLE (Edgar, 2004) as implemented in Geneious. PRMT sequences of several North American *Ischnura* species were downloaded from the GenBank, and used as outgroups in the phylogenetic analyses. For the COII dataset, we sequenced this gene in samples of *Ischnura hastata* Say, 1839 (see Table 1), but also downloaded sequences of several coenagrionids from the GenBank.

Phylogenetic relationships were reconstructed using maximum likelihood (ML). Heuristic searches were carried out using the randomized accelerated maximum likelihood algorithms implemented in RaxML-HPC2 (Stamatakis, 2006; Stamatakis, Hoover, & Rougemont, 2008), through the CIPRES web portal (<http://www.phylo.org>). The ML analysis was run under the GTR + G model, and clade support was assessed through 1000 bootstrap replicates.

We also evaluated clade probabilities through a Bayesian inference (BI) approach in MrBayes v3.2.3 (Ronquist & Huelsenbeck, 2003) in the CIPRES web portal. Searches were run for two million generations and were sampled at intervals of 1000 generations in three independent runs, using default priors and the GTR + I + G (for mtDNA data) and HKY + G (for nDNA data) substitution models, as selected by JModelTest 2 (Darriba, Taboada, Doallo, & Posada, 2012; Guindon & Gascuel, 2003). Burn-in samples (500 trees) were discarded and the remaining samples were combined to produce a 50% majority rule consensus tree, with bipartition frequencies equal to posterior probability values.

Genetic divergence between species was estimated using the uncorrected p-distances as calculated in MEGA v. 6.06 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013).

## Results

### Genetic and phylogenetic analyses

The final COII dataset consisted of 44 sequences of 593 bp length, with 306 polymorphic sites of which 279 were parsimony informative. The PRMT dataset consisted of 20 sequences of 502 bp length, with 182 polymorphic sites of which 170 were parsimony informative. All sequences have been deposited in GenBank, with accession numbers KT956137-KT956168 (Table 1).

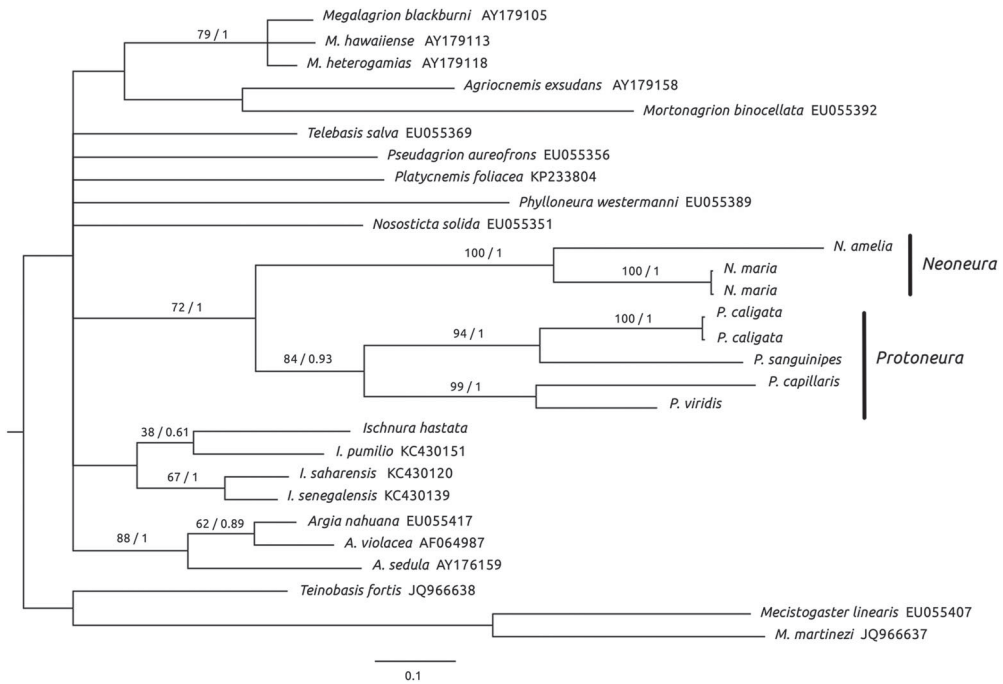


Figure 1. BI tree depicting the phylogenetic relationships between *Protoneura* and *Neoneura* species using COII. Numbers above branches indicate bootstrap/Bayesian posterior probability values, respectively.

Results of phylogenetic analyses were congruent for both mitochondrial and nuclear DNA; and also for BI and ML analyses, indicating that *M. caligata* belongs to the *Protoneura* clade (Figures 1 and 2). *P. sanguinipes* appears as the sister taxon of *M. caligata*, and the closest relative of *P. capillaris* is *P. viridis*. These relationships are supported by the high bootstrap and posterior probability values for both nuclear and mitochondrial DNA (Figures 1 and 2).

Interspecific distances for the COII ranged from 15.7% between *P. sanguinipes* and *M. caligata* to 23.1% between *N. amelia* and *M. caligata*. Genetic distances were lower for the PRMT locus, ranging from 1.4% between *P. sanguinipes* and *M. caligata* to 14.0% between *N. amelia* and *M. caligata* (Table 2).

### Notes on emergence and oviposition behaviour of *Protoneura caligata*

The stream Vegas Grandes is a forest stream, tributary of the river Caburní, 5–10 m wide, 0.20–1 m depth, with a bed formed by sand, gravel and boulders and with pools and rapids through the water course (Figure 3A). Other odonate species found in this river are *Protoneura capillaris*, *Enallagma coecum* Kolbe, 1888, *Scapania frontalis* Burmeister, 1939, *Hypolestes trinitatis* Gundlach, 1888, *Progomphus integer* Hagen in Selys, 1878, *Cannaphila insularis funerea* Carpenter, 1897 and *Dythemis rufinervis* Burmeister, 1839.

On 17 June 2012, a newly emerged female of *Protoneura caligata* completely off the exuvia and about 3.5 cm above the water surface was detected at 10:25 am. The spreading of the wings culminated at 11:03 am and that of the abdomen at 11:12 am. The whole process of the emergence finished at 11:17 am, when the female made the first flight to the upright canopy, approximately 15 m above the water surface. The general coloration of the teneral individual was light green, almost transparent (Figure 3C, D). The environmental temperature and relative humidity at the end of the emergence were 24.8°C and 74% respectively.

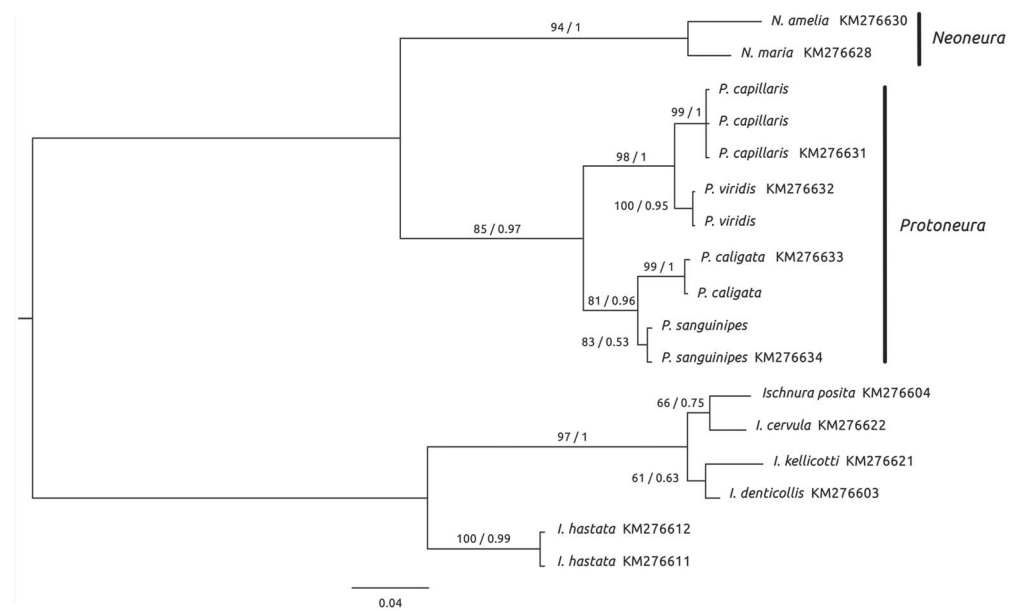


Figure 2. BI tree depicting the phylogenetic relationships between *Protoneura* and *Neoneura* species using PRMT. Numbers above branches indicate bootstrap values/Bayesian posterior probability values, respectively.

Table 2. Mean evolutionary divergence (uncorrected p-distance) between the Protoneurinae species included in this study. Distances are presented as percentages for both the PRMT (above diagonal) and COII (below diagonal) loci.

	P. caligata	P. capillaris	P. sanguinipes	P. viridis	N. maria	N. amelia
Protoneura caligata		6.0	1.4	5.3	13.3	14.0
P. capillaris	19.7		4.6	1.4	13.6	14.3
P. sanguinipes	15.7	21.9		3.9	12.9	13.6
P. viridis	18.4	14.3	20.2		13.3	14.0
N. maria	21.5	22.3	22.5	21.8		3.7
N. amelia	23.1	22.8	23.3	20.7	15.3	

The first pairs of *P. caligata* in tandem were detected from 12:08 pm onwards. As previously described by Westfall (1964), oviposition was observed to occur in tandem (Figure 4A), although we also observed females ovipositing alone (Figure 4C). During the oviposition in tandem, the male remained in a vertical position with the wings motionless ( $n = 16$ ) or changed from this behaviour to settling on the oviposition substratum ( $n = 2$ ). On three occasions the female oviposited submerged, either in tandem with the male ( $n = 2$ , Figure 4B) or alone ( $n = 1$ ), and we also observed mating pairs ovipositing together in the same plant stem (three pairs observed in group oviposition on 16 June, and three on 17 of June; Figure 4D). The registered oviposition period ranged between 15 and 20 minutes. Oviposition occurred on leaves and stems of *Cyperus* sp., *Hedychium coronarium* and in the submerged stems of *Mikania micrantha*.

## Discussion

Our results of nuclear and mitochondrial DNA sequencing shown that *Microneura* is a junior synonym of *Protoneura*; which confirms the results of the morphology-based phylogeny from





Figure 3. (A) Habitat of *Protoneura caligata* in Cuba. Stream Vegas Grandes, Topes de Collantes. (B–D) Sequence of emergence of a female of *Protoneura caligata* in Vegas Grandes, Cuba; in June 2012. (B) Female completely off the exuvia, as it was first detected. (C) Teneral individual after completing the spreading of the wings. (D) Female after emergence is completed, before flying to the upright canopy. All photos by M. Olalla Lorenzo-Carballa.

Pessacq (2008) and provides evidence of the diversification of the genus *Protoneura*, in which *caligata* is a species that gathers a number of particular and distinctive morphological characteristics that were the basis of its recognition (until now) as a species of a different genus. Our results stress also the importance of using an integrative approach, based on molecular and morphological data, to establish species boundaries (Dayrat, 2005); which is a crucial issue for some odonate groups, in which the use of morphological data alone cannot elucidate their taxonomy.

The results of our phylogenetic analyses show *P. sanguinipes*, an endemic from Dominican Republic, as the sister taxon to *P. caligata*, whereas *P. capillaris*, which is another Cuban endemic, appears more closely related to *P. viridis* from Jamaica. Donnelly (1989) and Meurgey and Picard (2011) hypothesized about the relationships between the Antillean *Protoneura* and their continental circum-Caribbean congeners. According to these authors, two groups can be

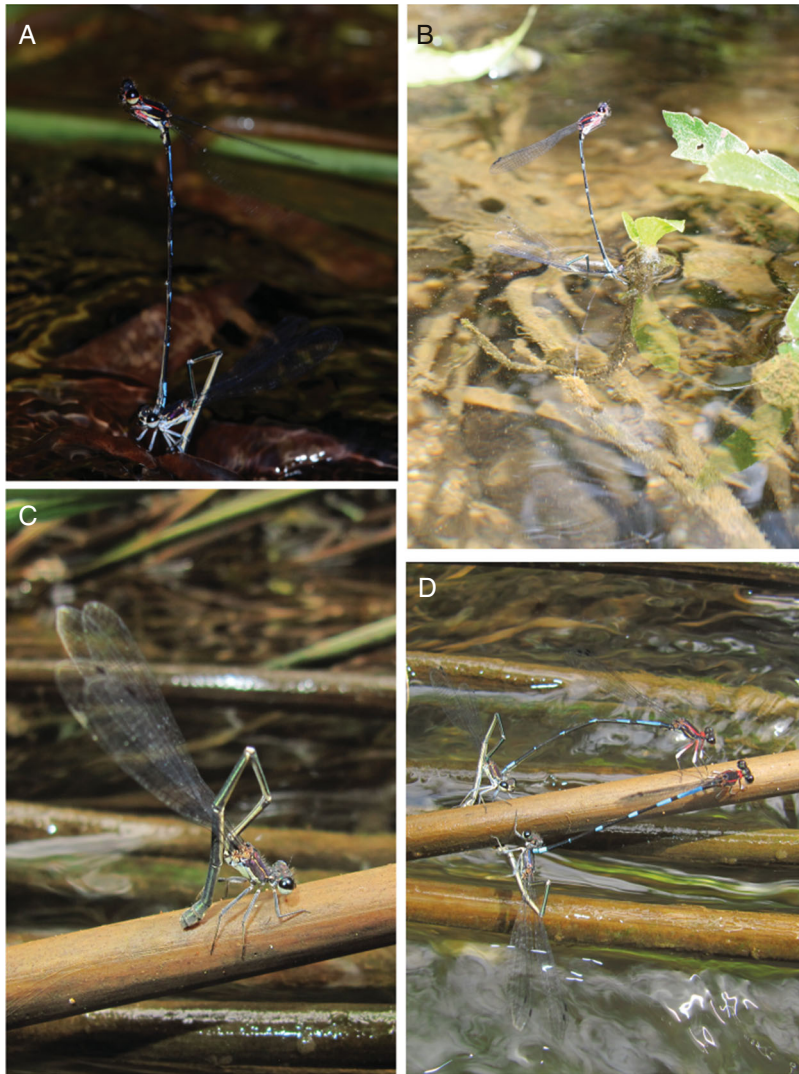


Figure 4. Oviposition of *Protoneura caligata* in Vegas Grandes, Cuba. (A) Pair ovipositing in tandem (Photo by Arjen Van't Hof). (B) Female ovipositing underwater (Photo by M. Olalla Lorenzo-Carballa). (C) Female ovipositing alone (Photo by Yusdiel Torres-Cambas). (D) Aggregated oviposition (Photo by Y. Torres-Cambas).

recognized on the basis of male anal appendages and female mesostigmal plate morphology. The “tenuis” species group (Meuruey & Picard, 2011), includes *P. ailsa*, *P. romanae*, *P. viridis* and *P. capillaris*, and has *P. tenuis*, a species found in Trinidad and Tobago, Venezuela, Peru, French Guiana and Brazil, as its most recent common ancestor. The second group, named “corculum” (Meuruey & Picard, 2011) after the suspected most recent common ancestor *P. corculum* (from Mexico and western Cuba), includes the species *P. sanguinipes* and *P. dunklei*. Our results confirm that *P. capillaris* and *P. viridis* are sister species, but also contribute with new data to this hypothesis, by suggesting that *P. caligata* should be included in the “corculum” group, given its phylogenetic relationship with *P. sanguinipes*. This would be in agreement with the hypothesis of Donnelly (1989) about a west–east (México–Cuba–Hispaniola) colonization/speciation sequence; since *P. corculum* occurs in western Cuba and *P. caligata* is restricted to the central part of this island. Further sampling is needed to confirm this hypothesis, as well as to clarify



the still uncertain monophyly of the genus and to better understand the relationships between the different *Protoneura* species.

Finally, our observations on the oviposition behaviour of *P. caligata* add some new information to those by Westfall (1964), who reported only oviposition in tandem for this species, and thus we provide evidence of the variability of this behaviour in this species. The oviposition behaviour observed in *P. caligata* is similar to *Neoneura maria* (Y. Torres-Cambas, personal observations), but different to what is observed in other species of the genus, e.g. *Protoneura cara* Calvert, 1903, *P. romanae* Meurget, 2006 and *P. capillaris*, in which oviposition occurs in tandem, with males also adopting the vertical position as in *P. caligata*, but they keep flapping the wings, and generally the males of these species do not settle during oviposition, neither do the females oviposit alone (González-Soriano, 2001; Meurget & Picard, 2011; Trapero Quintana, Torres-Cambas & González Soriano, 2005). As also observed in *P. caligata*, tandem oviposition is the predominant mode in *Protoneura amatoria* Calvert, 1907 although females can oviposit alone (Larison, 2007). The oviposition substrates used by *P. caligata* are similar to those used by *P. capillaris*, *P. cara*, *P. sanguinipes*, *P. romanae* and *N. maria* (González-Soriano, 2001; Meurget & Picard, 2011; Trapero Quintana et al., 2005; Y. Torres-Cambas, personal observations).

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